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Summary Statement on the Enzyme System

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PRODUCTION OF ENZYME

Status of Work

The best sources of extracellular cellulase are the *Trichoderma viride* mutants QM 9123 and QM 9414; the best medium for cellulase production is that developed by Mandels and Reese with 0.5-1.0% pure or waste cellulose as carbon source. Highest cellulase yields in shake flasks, or in 10-liter batch fermentors are: for CMC, about 100 units per ml; for filter paper, about 2 units per ml (or a FP activity of 5.0 mg of glucose per ml of enzyme per hr); and for cotton, an activity of about 9 mg of glucose per ml of enzyme per 24 hr. Culture broths contain about 2 mg of protein per ml. Continuous fermentations have been established that give lower concentrations of enzyme, but higher yields (i.e., enzyme per day) than obtained in batch processes. The cost of producing enzyme is low as compared to the cost of milling the substrate.

The assay procedure using filter paper as cellulose source is being adopted as most satisfactory for routine use in the cellulose hydrolysis program (see Dr. Mandel's paper for details, p. 81). This method, with minor differences, is in use in those laboratories most active in achieving cellulose to glucose conversion, e.g., Natick, Berkeley, and Peoria.

Plans for Future Activity

The greatest problem to be overcome in developing an economic conversion of cellulose to glucose is the limited extent and slow rate of hydrolysis of untreated substrates. The milling or other methods used to increase the digestibility of the crude materials are a major cost (4¢/lb).

Biotechnol. & Bioeng. Symp. No. 5, 77-80 (1975)

Increases in enzyme potency are important in that they may lessen the need for the present extensive grinding. Two major programs should be pursued, aimed at increasing enzyme yields.

1) Optimize conditions for enzyme production in fermentors at the prepilot plant level. It is particularly important to obtain a continuous process for enzyme production. Such work requires batteries of fermentors with a high degree of automatic control (pH, aeration, agitation, temperature, etc.).

2) A mutation program should be aimed at obtaining mutants having one or more of the following characteristics: a) increased yield of enzyme, b) increased stability and activity at higher temperatures, c) ability to attack substrates that have not been milled, and d) ability to produce enzyme in the presence of sugars. Basic research on the induction process should be continued in conjunction with the mutation program.

Novel means of obtaining active cellulase preparations should be sought by testing strains of thermophiles and anaerobes. Mixed cultures may also prove to be worth trying. The need for maintaining aseptic conditions requires examination into these possibilities.

Another necessity is a cellulase assay that is quantitative and generally accepted so that communication between laboratories may be more meaningful.

MODE OF ACTION OF CELLULASE SYSTEM

Current Status

The enzyme system of *Trichoderma viride* has been examined more extensively than any other. The majority of its components have been found to be glycoproteins. Amino acid analyses, molecular weights, and specific activities have been determined for several of the resultant purified enzymes. pH and temperature optima are well established. The current trend is to consider the cellulase system to be composed as follows.

1) Endo-β1,4 glucanases, the old C_X, are present with several components varying in degree of randomness. One of these may be the enzyme that acts first on "crystalline" cellulose. A specific activity of >60 μ/mg has been established (est. of ETR using carboxymethyl cellulose 50T).

2) Exo-β1,4 glucanases are present in varieties: a) β1,4 glucan glucohydrolase, removing single glucose units from the nonreducing end of the chain and b) β1,4 glucan cellobiohydrolase (CBH) removing cellobiose units from the nonreducing end of the chain. This CBH is currently being equated with the old C_1 enzyme by many investigators. The pure CBH component has a specific activity of 0.7 μ/mg (vs. Walseth cellulose), and is the component having the greatest affinity for cellulose.

3) β-glucosidase (cellobiase) having a specific activity of 70 μ/mg (vs. cellobiose) is also present. The relationship of this enzyme to the glucohydrolase has not yet been clarified.

For complete activity on crystalline cellulose, it is necessary that several of the preceding components be present. Components isolated from different organisms act synergistically, as do the components isolated from the same organism.

Proposed Activities

The practical problem of converting cellulose into glucose can move forward on the basis of the current concepts of "cellulase." However, several topics require further careful study, such as:

1) The endo-β1,4 glucanases (C_X) having different physical properties (size, charge, composition). A more complete examination of the action of each component, may elucidate its role in hydrolysis.

2) Does a physical complex of components exist at the substrate surface? (Current evidence indicates a lack of complex in the free solution.) How many distinct polypeptides are in the complex?

3) Assay procedures should be standardized for each enzyme type. A number of substrates should be employed to define the specificity of the system. Amounts of protein should be specified, and international units (IU) used.

4) Methods for characterization of the insoluble products (like the soluble ones) are required.

RELATED ENZYMES

Related enzymes are those which may aid cellulases in one way or another to convert complex cellulosic residues to glucose.

Lignases

Delignification, by chemical means, increases the rate of hydrolysis of the cellulose present in woody materials. In nature, many fungi degrade and use lignin, but, as yet, no enzymes have been isolated and demonstrated to have this ability. However, the chemical nature of lignin is now fairly well determined, and the changes brought about by fungal action are partly understood. With the recent availability of ^{14}C-lignin, an assay procedure for degradation is available which is rapid, unequivocal, and sensitive.

The first objective is to discover organisms which can rapidly degrade lignin, and to obtain from these the various enzymes responsible. Strain improvement, induction studies, and optimization of enzyme production will follow. Finally the characterization of the purified enzymes must be achieved; their interrelationships defined, as well as their connection, if any, with the cellulase system.

While our immediate interest in lignases is as a method for increasing the digestibility of cellulosic materials, lignin breakdown products may be of great value in themselves, e.g., as chemicals and as fuels.

H_2O_2 Producing Enzymes

A number of microorganisms are known to produce hydrogen peroxide, which, especially in the presence of Fe^{++}, has the ability to act on crystalline cellulose to bring about changes resembling those of enzymatic hydrolysis (e.g., alkali swelling, alkali solubility, decrease in degree of polymerization). Is this a method by which some organisms utilize cellulose? Probably not, by itself, since the products of peroxide action are oxidized sugars, but it is conceivable that peroxide action may be coupled with action of certain of the cellulase components (e.g., C_x).

The fact that many cellulolytic organisms apparently secrete little or no cellulase (unlike *Trichoderma*) makes it imperative that we continue to search for alternate means of cellulose degradation. H_2O_2, and the super-oxide anion radical, are two approaches meriting further work.

(*Note*: The above information is a concensus arrived at in discussion by the whole group. No one individual, even the writer, accepts all the conclusions. The responsibility for inserting specific values was assumed by the author.)